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CONSTRUCTION OF MUTANTS FOR A CYSTEINE ACCESSIBILITY ASSAY FOR THE YEAST SEC61 COMPLEX

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CONSTRUCTION OF MUTANTS FOR A CYSTEINE ACCESSIBILITY ASSAY FOR THE YEAST SEC 61 COMPLEX

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ABSTRACT

The Sec 61 complex in eukaryotes is a passive pore in the endoplasmic reticulum (ER) membrane that permits a secretory protein to enter the ER membrane or lumen. To facilitate understanding of channel opening and closing, and especially to help determine the location of the plug domain relative to key cysteine residues, several yeast Sec 61 mutants were constructed by site-directed mutagenesis of cysteine residues at positions C121, C150, and C373. The mutants expressed Sec 61 and showed growth defect phenotypes.

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BACKGROUND

Protein Secretion

Protein secretion, the process whereby proteins are assembled into membranes or secreted into the matrix, has long been of interest to researchers (Rapoport, 2007). This process results from the interaction of two major organelles, the endoplasmic reticulum (ER) and Golgi apparatus, transporting materials via vesicles. Early work on secretion includes the finding of George Palade that secretory proteins cross the ER membrane before being transported to the plasma membrane at the cell surface via vesicles (Palade, 1975). And in 1975, Gunter Blobel suggested that the aqueous TM channel helps mediate protein transport through the rough ER (RER) membrane (Blobel and Sabatini, 1971). Blobel later determined that secretory proteins contain an N-terminal signal sequence that directs the translocation of secretory proteins across the RER membrane (Blobel and Dobberstein, 1975).

Endoplasmic Reticulum

The ER is the largest organelle in eukaryotes (by volume), and functions in a variety of biosynthetic reactions, including the synthesis of secretory proteins, calcium ion storage, membrane integrations, synthesis of steroids and phospholipids, and post-translational protein folding and modification (Matlack *et al.*, 1998). The ER membrane initially forms from the nuclear envelope, and extends throughout the entire cytoplasm. It is a single membrane organelle containing a continuous intraluminal space. The ER is divided into the rough ER (RER) (named after the rough granulated appearance due to

the presence of membrane-bound ribosomes), and the smooth ER (SER), and the lumenal flow is from the RER to the SER (Voeltz *et al*., 2002).

The RER is the site of secretory protein synthesis and membrane integration. Protein translocation occurs primarily in this region of the ER. The SER has different functions depending on the cell type, and is found in all cells involved in packaging proteins for export to the Golgi. SER functions include calcium ion storage and secretion in muscle, production of steroid and phospholipids, or detoxification of hydrophobic substances, depending on the cell type (Voeltz *et al*., 2002).

ER Translocation Channel

Most proteins are transported through the ER membrane by a translocation channel (Rapoport, 2007). The translocation channel was discovered by genomic mutation screening, electrophysiology, and florescence quenching experiments (Menetret *et al*., 2000). The channel is formed from a conserved heterotrimeric protein complex (**Figure-1**) whose channel allows the transfer of the secretory polypeptide chain across, or integration into, the ER membrane. Targeting to the channel is facilitated by the presence of the N-terminal hydrophobic signal sequence and its bound signal recognition particle. Removal of the signal sequence by signal peptidase in the ER membrane reinitiates protein synthesis in the ER lumen. For membrane proteins, once their hydrophobic transmembrane domains are synthesized, they enter the ER membrane through an opening in the channel, and are released into the lipid phase (Van den Berg et al., 2004).

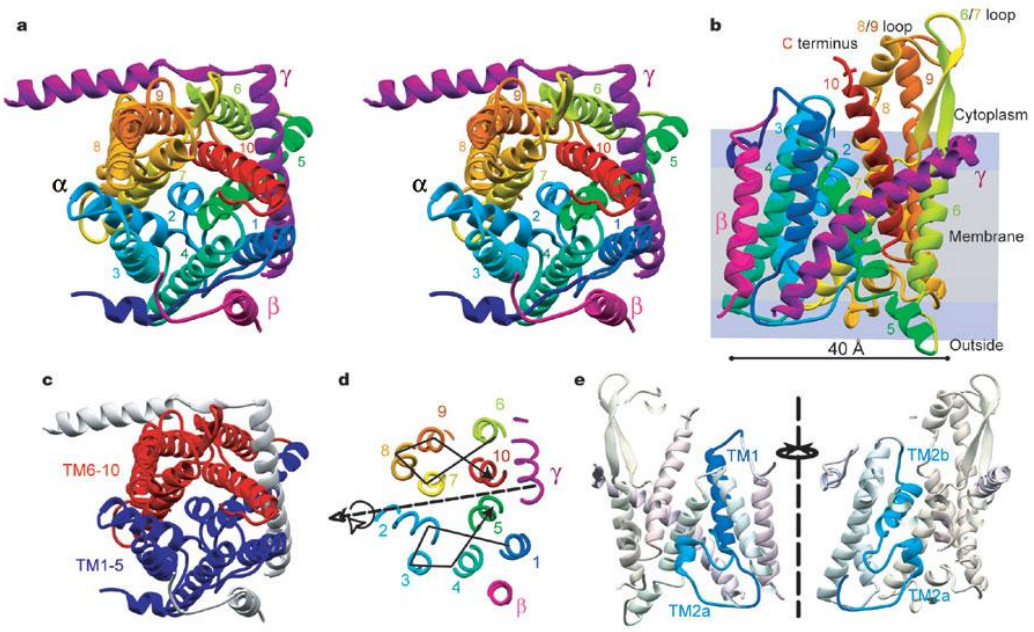

Figure-1: Structure of the ER Translocation Channel. **A**, Stereo view from cytosolic view of the structure. The numbers represent the trans-membrane segment number. The red to blue helix is the ∝-subunit, the β-subunit is pink, and the ɣ-subunit is magenta. **B** The back of the structure with the phospholipid head group and hydrocarbon regions of the membrane shown in blue and grey in the background. Cytosolic loops are indicated in the structure. **C** Top view with the N- and C-terminal halves of the ∝-subunit in blue and red, respectively. **D** The top view of the membrane. **E** Slab views of the structure. The foreground are removed with TM1 in dark blue, TM2a and TM2b in sky blue. (Van den Berg *et al*., 2004).

The ER translocation channel is formed from a conserved heterotrimeric membrane complex, either Sec61 (in eukaryotes) or SecY (in bacteria and archaea). The α-subunit is the largest subunit of this channel, consisting of 10 transmembrane domains, with its N- and C-termini in the cytosol. The β-subunit in eukaryotes and archaea spans the membrane once, while in eubacteria it spans the membrane twice. The γ- subunit spans the membrane once (Osborne *et al*., 2005). Genetic knock out experiments performed in *S. cerevisiae* and *E. coli* have shown that both the α and γ subunits are

required for cell viability. The α- subunit forms the pore of the channel, and contains the docking site for the signal sequence (Van den Berg *et al*., 2004). The Sec 61 channel is a passive pore that allows polypeptides to enter the ER lumen. The channel needs cytosolic partners for translocation to occur, and based on the type of partner there are three different modes of translocation: two modes in post-translational translocation, and one in co-translational translocation (Rapoport, 2007).

Co-Translational Translocation

The ribosome is the primary partner involved in protein co-translational translocation, found in all cells. This mode of translocation is used for some types of secretory proteins and for the synthesis of integral membrane proteins (**Figure-2**) (Rapoport, 2007).

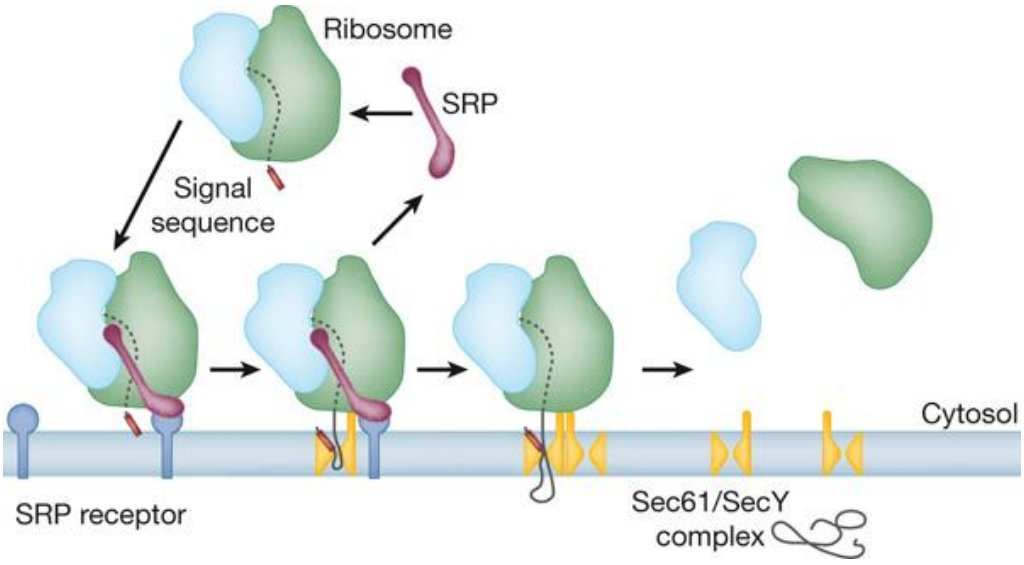

Figure-2: Diagram of Co-Translational Translocation. In this process, a ribosome initially synthesizes a portion of the secretory protein containing the signal sequence. Signal recognition particle (SRP) binds the signal sequence halting translation. The SRP then binds the SRP receptor in the ER membrane docking the ribosome. The ribosome then associates with the Sec61 complex, which helps cleave the signal sequence to resume translation of the secretory protein and its release in the ER lumen (Rapoport, 2007)

This process begins in the targeting phase, where the cytoplasmic signal recognition particle (SRP) recognizes the transmembrane span or signal sequence of a growing secretory polypeptide chain, temporarily halting its translation. The complex containing the ribosome, mRNA, and SRP is then targeted to the ER membrane when the SRP and ER SRP-receptor interact. This docking leads to interaction between the ribosome and the translocation channel (Rapoport, 2007). Translation resumes, and the protein can enter the lumen or the membrane. Although the movement of the polypeptide in the channel is independent of nucleotide hydrolysis, movement to the membrane is mediated by GTP hydrolysis (Osborne *et al*., 2005).

Post-Translational Translocation

The second type of translocation, post-translational translocation, occurs when proteins are transported after synthesis (**Figure-3**). In yeast, about 60% of proteins are translocated by this mechanism. Yeast and bacteria often use this pathway because of different paces of translation and translocation (Rapoport, 2007).

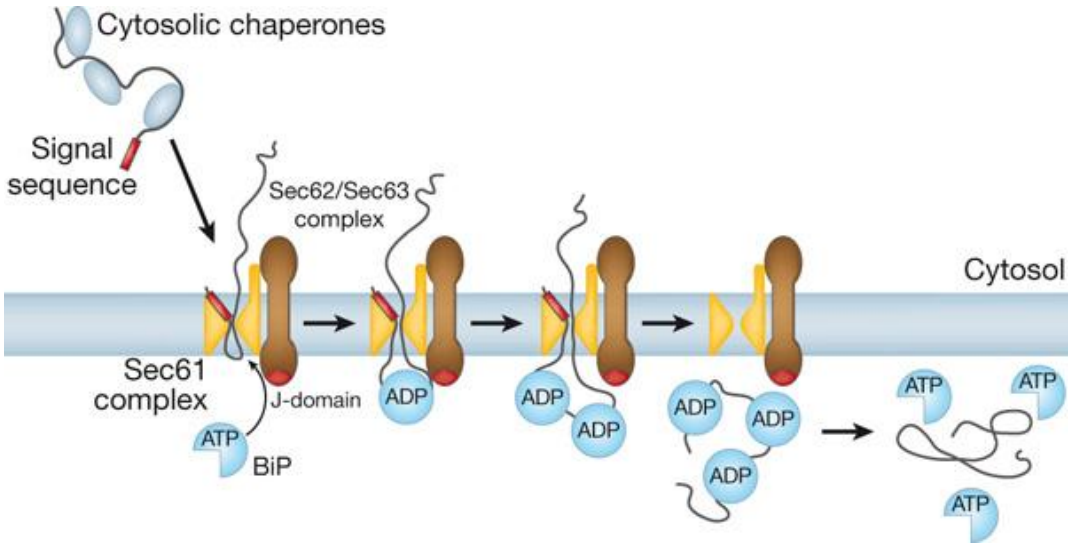

Figure-3: Diagram of Post-Translational Translocation in Eukaryotes. This process is similar to the previous diagram, except the secretory or membrane protein arrives at the RR membrane already synthesized. (Rapoport,2007)

In yeast and most eukaryotes, this process begins by the binding of cytoplasmic chaperones, such as HSP70, to the fully synthesized secretory or membrane protein. Then this complex docks with the ER tetrameric Sec62/63 complex in the ER membrane, and the protein is released from the cytosolic chaperons. After the polypeptide is inserted into the channel, a ratcheting mechanism causes translocation and prevents the protein from sliding back and forth by Brownian motion. Binding to the BiP inside the lumen of the ER prevents the polypeptide from moving back into the cytosol. Closing of the Sec61 peptide-binding pocket around the peptide occurs when ATP-bound BiP interacts with the J-domain of Sec 63.BiP activated by the J-domain interacts with any polypeptide segment that appears from the channel into the lumen. The process continues until the polypeptide chain has navigated into the channel. In the final stage, ATP is hydrolyzed, and the peptide-binding pocket opens and releases BiP (Rapoport, 2007).

In bacteria, post-translational translocation involves the Sec Y channel and the cytosolic ATPase SecA, and this applies to most of the secretory proteins (**Figure-4**). Sec A contains several domains, including two nucleotide binding folds (NBF). After the Sec Y undergoes a conformational change with the ATPase, the polypeptide is pushed through the channel (Osborne *et al*., 2005).

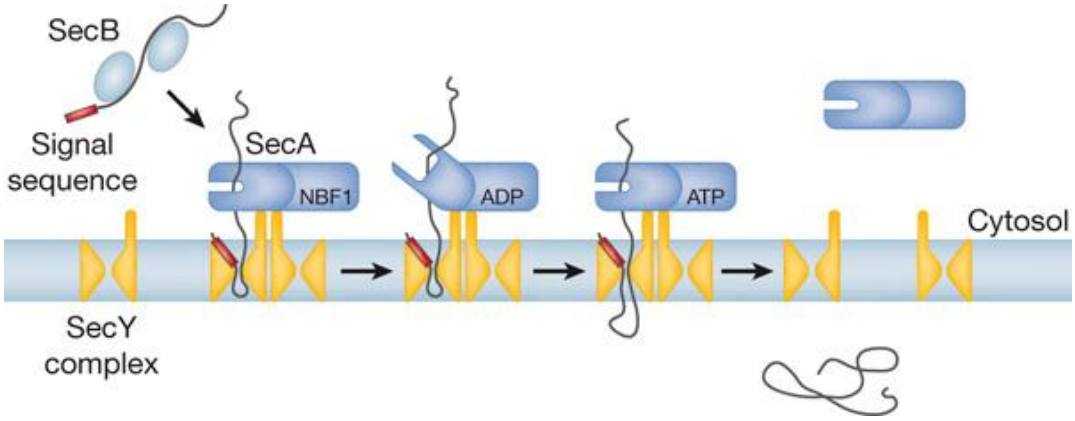

Figure: 4: Diagram of Post-Translational Translocation in Bacteria. This process is similar to that described for eukaryotes, except the translocation channel is Sec Y, and the ATPase is located in the cytoplasm. (Rapoport,2007)

Open State of the Channel

The translocation channel must be in the open state for secretory proteins to transport across the membrane (Matlack *et al.*, 1998). Channel opening involves two steps, starting with the binding of the channel to a partner used in either mode of translocation. Prior to ribosome binding, a plug occurs in the center of the channel. Ribosome binding occurs with a cytosolic loop in the carboxyl half of the channel, causing a temporary displacement of the plug domain, and a continuous opening and closing of the lateral gate (Rappaport, 2007).

The second step involves the hydrophobic segment of a signal sequence entering the lateral gate. Experiments have shown that creating a defective signal sequence can destabilize the closed channel (Rapoport, 2007). Further information about how the channel opens have also been proved by analysis of channel structures, biochemical characterization of translocation intermediates, molecular dynamics simulations, and *in vivo* and *in vitro* analysis of structure-based Sec61 and SecY channel mutants.

In mammals, the ribosome binding site on Sec61 was mapped to Loop 6 and Loop 8, both of which are exposed cytoplasmically. Upon binding, the channel undergoes a conformational change, so the signal sequence is inserted into the sequence-binding (SSB) site (Zimmer et al., 2008).Three structures with different SecY lateral gate domains have been characterized from *M. jannaschii*, *T. thermophilus*, and *T. martima*, and have shown the partial open state of the channel (**Figure-5**). The structure of the plug domain in *M. jannaschii* opens in the front of the lateral gate with two α subunits. The crystal structure of this plug domain shows an L shaped structure, with the tip of the loop parallel to the membrane surface. Comparing to loop 6, loop 8 is closer to the membrane bilayer. The closed conformational shape of *T. thermophiles* SecY-E is similar to the *M. jannaschii* SecY-E beta structure. The binding of anFab antibody to the tip of loop8 causes a separation of the cytosolic end of the lateral gate. For the *T. maritime* plug domain, Sec YEG –Sec A complex has a conformational change when adding a non-hydrolysable ATP analog. The binding of SecYEG and SecA results in separation of the cytosolic and exofacial portion of the SecY lateral gate (Mandon *et al.,* 2009).

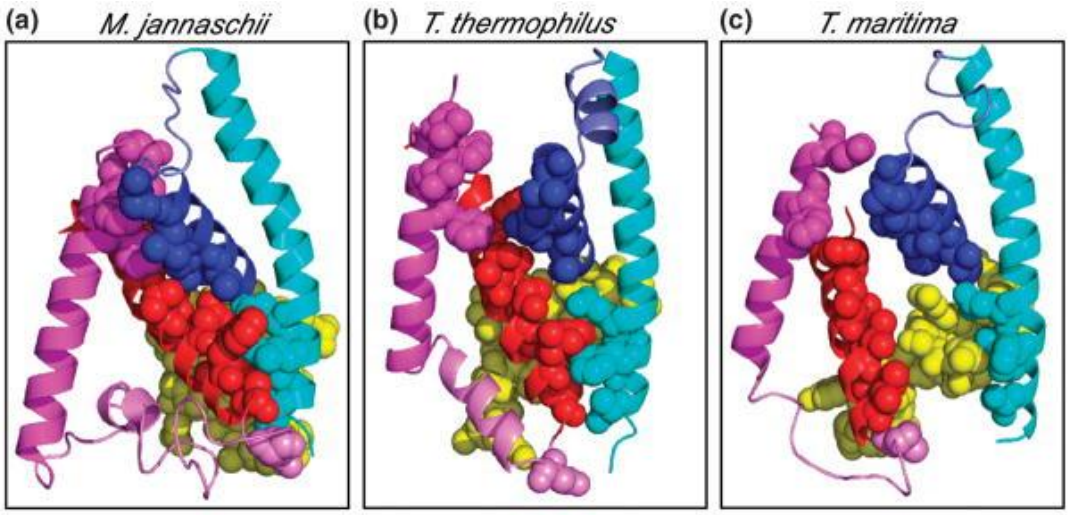

Figure 5: Diagram of Three Known Structures of the Sec Y Lateral Gate Domain. Shown are the known structures for the lateral gate domain of (a) *M. jannaschii* ; (b), *T. thermophilus* ; and (c) *T. maritima*. (Mandon *et al.,* 2009).

Membrane barriers for ions and small molecules are maintained in the SecY complex during protein translocation. For the closed state of the channel, a binding partner is not needed for the SecY molecule. The plug domain and pore ring work together keeping the channel closed for small molecules. Mutating the pore ring could affect interactions that keep the plug in the center of the molecule, and moving the plug could change the size of the pore ring. In previous experiments, modifying the cysteine residue in the plug and the pore ring caused the channel to open transiently for the SecY complex. With disulfide bridge formation, the plug was moved out and the channel opened permanently. Similar experiments have not yet been performed for eukaryotic Sec61. The Sec61 complex in eukaryotes is similar to SecY by sequence conservation (Saparvo *et al*., 2007).

PROJECT PURPOSE

To further understand how the ER Sec61 protein-conducting channel opens during protein translocation, new channel structures must be analyzed, including translocation intermediates, and *in vivo* and *in vitro* synthesized Sec61 mutants. For this project, novel Sec61 mutants were designed based on the known structure of the homologous *M. janaschii* SecYEb complex. The full open state for the molecule is unknown. A possible disulfide bond could be formed between a cysteine residue in the plug helix and the periplasmic tail of SecE, but it is unknown whether this cysteine residue is accessible for bond formation in the open state of the channel (Mandon *et al.*, 2009). The purpose of this project was to design and synthesize various SecYEb channel domains and mutants to help investigate the molecular mechanisms of channel opening.

METHODS

Plasmid Cloning

Sec61 Mutagenesis and PCR

Four different yeast Sec61 mutants were created by PCR site-directed mutagenesis by other personnel in our laboratory. The four mutants created are listed in a Table at the beginning of the Results section.

Purification of PCR Product

Sec61 PCR amplicons were purified from agarose gels using the QIAquick Gel Extraction kit (Qiagen). Each amplicon band in an agarose slice was mixed with 850 µl of the provided QG buffer, and incubated at 55ºC for 15 minutes. Then 250 µl of ethanol was added to each sample and mixed. The sample was transferred to a 2ml collection tube, and centrifuged for 1 minute to pellet the DNA bound to beads. The pellets were washed with 750 µl of Buffer PE, then microcentrifuged for 1 minute. The DNA was eluted from the beads using 43 µl of sterile dH2O.

Restriction Digestion of Amplicon Bands and Plasmids

Purified amplicon and plasmid DNAs were digested with HindIII according to the following Table:

Component	Amplicon Digestion	Plasmid Digestion
10X Buffer-2	4 µl	2 µl
BSA	0.4 µl	0.2 µl
HindIII	2.0 µl	1.0 µl
Sterile dH_2O		10.8 µl
Amplicon DNA	33.6 µl	
Plasmid DNA pRS316		6.0 µl
Total Reaction Volume	**40** µl	**20** µl

The reactions were incubated for 2 hours at 37ºC, then 0.5 µl of calf intestinal phosphatase (CIP) was added to the plasmid reaction (to dephosphorylate the 5' end to help prevent self-ligation)and the reaction was incubated for an additional 30 minutes at 37ºC. Without inactivating either enzyme, the reactions were directly electrophoresed on agarose gels to determine how much of the Amplicon and plasmid to add to the ligation mix.

DNA Ligations

DNA ligations were set up according to the following:

Ligation	Amplicon Digestion
Amplicon HindIII Digestion	x- µl
Plasmid HindIII Digestion	x- µl
T4 DNA Ligase	2.0 µl
Sterile dH_2O	x- µl
10X T4 Ligase Buffer	2 µl
Total Reaction Volume	**20 µl**

The tubes were incubated at 16ºC overnight, using a thermocycler.

DNA Transformation into E. coli

Following ligation, 3 µl of the ligation reaction was added to a separate centrifuge tube with 60 µl of thawed competent *E. coli* cells previously made competent by lab

personnel. These tubes were incubated on ice for 25 minutes, and were then placed into a 42˚C water bath for 30 seconds. Next, the cells were again incubated on ice for two minutes before 500 µl of LB media was added. Then, the cells were incubated at 37˚C or one hour. Following this incubation, 350 µl were spread on an LB-Ampicillin plate and incubated at 37˚C overnight.

Colony PCR

If *E. coli* colonies grew after the transformation, ~ 20 different colonies were randomly selected, picked with a toothpick, and mixed with 25 µl sterile water to make a suspension that was used as template. Each toothpick was placed in LB- Ampicillin media and grown overnight at 37°C. Each colony PCR reaction contained the following:

PCR reaction	Amount
Sterile dH_2O	14 µl
10 X Taq Buffer	2.5 µl
dNTP	2.5 µl
Taq Polymerase	1 µl
Sec61 Forward Primer	1.25 µl
Sec61 Reverse Primer	1.25 µl
Template	2.5 µl
Total Reaction Volume	**25 µl**

The tubes were microcentrifuged, then mineral oil was added to each reaction, and the tubes were placed in a thermocycler, and run as following:

PCR Program	Temperature	Time (Minutes)
Denaturation	95 ºC	5
Denaturation	94ºC	0.5
Anneal	50 ºC	0.5
Extension	72 ºC	2.5
Repeat Step 2-4		30 times

	72 ºC	5
	4 ºC	At least 10 minutes

Plasmid DNA Isolation

Plasmid DNA was isolated from overnight *E. coli* cultures using a QIA Prep Spin MiniPrep Kit (Qiagen). DNA pellets were resuspended in Buffer P1, then 250 ul of Buffer P2 was added to the resuspension and mixed. Then 350 ul of Buffer N3 was added, and the mixture was centrifuged for 10 minutes. The supernatant was transferred to a QIA prep spin column, centrifuged for 60 seconds, then 0.75 mL of ethanol containing Buffer PE was added. The column was centrifuged for 1 minute, and the flow through was discarded. The spin column was centrifuged for an addition minute, then the DNA was eluted with 50 ul of sterile water.

Plasmid Transformation into Yeast

Yeast cultures (WT strain V5) were grown in yeast medium +SSH and -SHH overnight at 30ºC, then plasmid DNA was isolated using a LiAc Method (lab TRAFO Solution Page) with two adjustment: the SS-DNA was not boiled, and the transformation mix consisted of the following instead of the amount stated in the procedure:

Transformation Mix	**Amount (μl)**
PEG (50% w/v)	240
1.0 M LiAc	36
ss-DNA	8
Plasmid	2
Sterile dH_2O	74
Total Reaction Volume	**360**

The mixture was added in the order listed in the table. After two 30 min incubations, one

at 30ºC then one at 42 ºC, the reactions were micocentrifuged for 15 seconds. The cell pellet was resuspended in 500 µl of sterile water instead of 1 ml, then plated on SD-AUT plates and incubated at 30ºC for two days.

Yeast Dilution Growth Assays

Sec61 plasmid containing yeast were grown overnight to an ideal optical density of less than 1 OD at 600 nM. Cell suspensions were diluted to 0.1 OD for a 50 ul sample, and then four 1:10 serial dilutions were prepared. 5 ul of the four diluted samples were plated on 2 YPD plates, then the plates were incubated one at 30ºC and one at 37ºC for two days.

Preparation of Yeast Whole Cell Lysates

Yeast cultures were grown overnight at 30ºC in 4 ml of YPD media. The cells were pelleted and resuspended in 200 µl of TCA Buffer. ~300 mg of glass beads was added to the tubes, then the tubes were vortexed six times for 30 seconds and put on ice for 30 seconds after each interval. The supernatant was transferred to a new tube, and the beads were washed 2 times with 100 µl of TCA Buffer. The supernatants were combined and centrifuged at 4ºC for 5 minutes. The pellets were resuspended in 200 µl of resuspension buffer, then incubated at 55ºC for 25 minutes.

Protein Quantification

Proteins were quantified using the Bradford Assay. A standard curve was generated using BioRad Reagent A and B with the following amounts of BSA in each tube: 0, 1 µl, 2 µl, 4 µl, and 6 µl. For each protein sample 2.5 µl was added to 22.5 µl of

water, then the BioRad reagent was added to each tube and incubated at room temperature for 15 minutes. The OD was taken at 750 nm.

Sec61 Western Blots

Protein samples were electrophoresed on 10% SDS-PAGE overnight at 6 volts, then transferred to membrane. The primary antibodies V5 and PGK were monoclonal purchased from Invitrogen, used at a dilution of 1:5000. The secondary antibody was anti-mouse IgG purchased from Pierce used at a dilution of 1:10000. Sec61 antibody was a gift to the lab, used at 1:5000. The secondary used for that antibody was anti-rabbit IgG purchased from Pierce and used at of 1:10000.

RESULTS

Yeast Sec61 Mutagenesis

To facilitate our understanding of the accessibility of substrates to the Yeast Sec61 translocation channel plug region, yeast cells carrying mutated forms of Sec61 plasmids were constructed and analyzed. The Sec61 positions selected for mutagenesis (see Table below) were based on the position of 3 key cysteine residues at positions 121, 150, and 373. All three cysteine residues were mutated for strains 170 and 172. The mutated I320C position in three of the strains is expected to provide ideal conditions for labeling with water soluble maleimide with or without the presence of ribosome. M69 and S72 (present in 170 and 172) are both in the plug domain area. Strain 138 was a control, mutated at unrelated sites.

Mutant Designation	Sec61 Residues Mutated
138	I86T, Q308A, I323A W326A, and L342A
pEM707	I320C
170	C121A, C150A, I320C, C373A, and M69C
172	C121A, C150A, I320C, C373A, and S72C

The first stage of this multi-step project involved constructing the four mutant Sec61 genes in plasmid DNAs, and confirming the presence of the mutation in the Sec61 gene. The mutants were created by site-directed mutagenesis of recombinant plasmids of the Sec61 gene by other personnel in the lab. Following PCR with mutated primers, the purified amplicons were digested with HindIII and ligated into similarly digested plasmid pRS316. Following transformation into yeast, colony PCR (**Figure-6**) was used to screen for positive clones containing the mutant of interest.

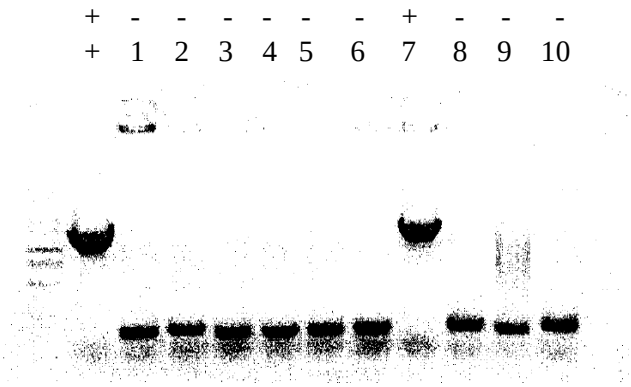

Figure-6: Example Gel for a Colony PCR. Shown is the colony PCR for clone pEM707. Lane 7 is positive.

Plasmid DNA was isolated from positive colonies, and mutations were confirmed by DNA sequencing. Mutated Sec61 plasmids were then transformed into yeast by using the LEU2 gene for selection by plating onto plates containing 5-fluroroorotic acid (5-FOA). If colonies are positive, URA3 gets shuttled out.

Effect of Sec61 Mutations on Growth

The potential difference in growth rates of the four prepared yeast Sec61 mutants were examined by plating serial dilution of cells onto YPD plates. In this experiment, positive and negative controls for each strain were based on the observation that Ssh1p is nonessential and its expression cannot suppress a null mutant. The control had an amino acid substitution at position R275 causing a growth defect in the absence of Ssh, but not in the presence of Ssh.

The first strain tested in growth experiments was pEM707, which had an amino acid substitution at I320C. **Figure-7** compares the growth of pEM707 with and without

SSH relative to control strain R275. The data confirmed there is a growth defect in pEM707 at either temperature. This mutant showed less growth without SSH (rows 5 and 6) compared to the presence SSH (rows 3 and 4) where more growth occurred. In the presence of SSH, pEM 707 showed a growth rate similar to R275. In the absence of SSH, pEM 707 showed less growth compared to WT, especially on the 37°C plate.

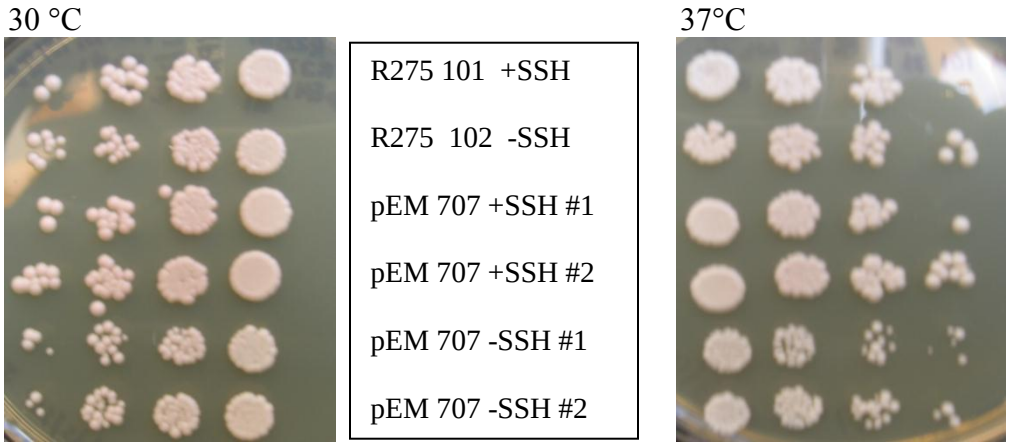

Figure-7: **Growth Analysis of Sec61 Mutant pEM 707**. pEM707 is mutated in Sec61 at I320C. Left panel was incubated at 30°C, and the right panel incubated at 37°C for 2 days. R275 was the wild-type control.

In **Figure-8**, mutant 138 containing Sec61 mutations at I86T, Q308A, I323A W326A, and L342A, was compared to R275 WT control. This strain also showed small growth defects relative to WT. In the absence of SSH (rows 5 and 6), the colonies were slightly smaller than the colonies in the presence of SSH (rows 3 and 4).

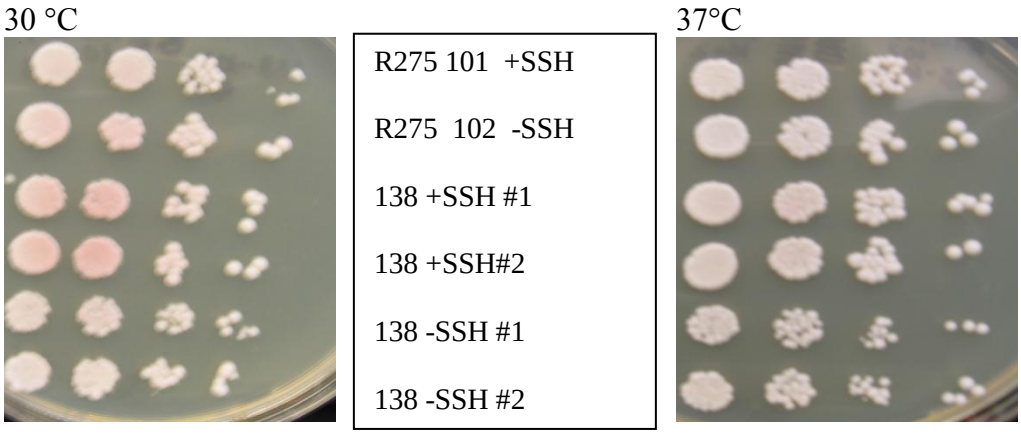

Figure-8: Growth Analysis of Sec61 Mutant 138. This mutant contains amino acid changes at I86T, Q308A, I323A W326A, and L342A. Left panel was incubated at 30°C and the right panel at 37°C for 2 days. R275 was the wild-type control.

Figure-9 shows the growth analysis of mutant 170, containing amino acid substitutions at C121A, C150A, I320C, C373A, and M69C. This mutant appears to show less growth in the presence of SSH (rows 5 and 6) relative to without SSH (rows 3 and 4).

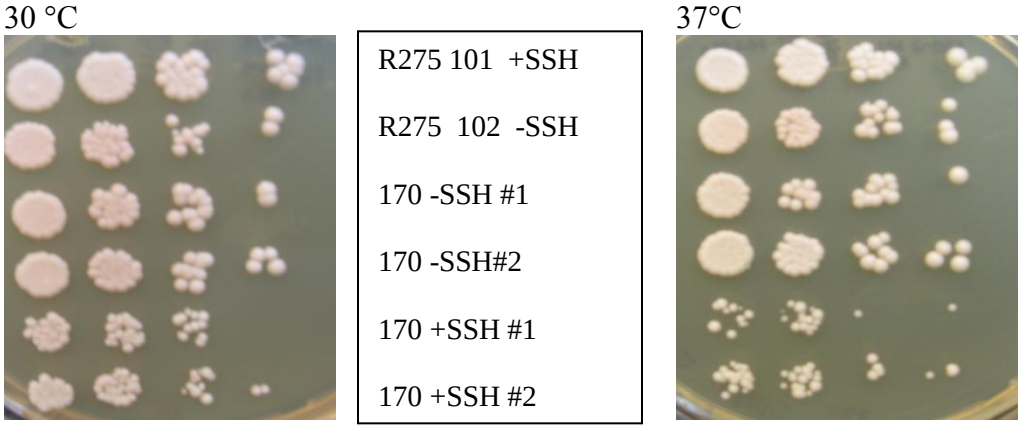

Figure-9: **Growth Analysis of Sec61 Mutant 170**. This mutant shows altered amino acids at positions C121A, C150A, I320C, C373A, and M69C. The left panel was incubated at 30°C and the right at 37°C for 2 days. Upper two rows contains R275 wild-type control.

Figure-10 shows the growth analysis of mutant 172 with amino acid substitutions at C121A, C150A, I320C, C373A, and S72C. This mutant appears to show a growth defect relative to WT.

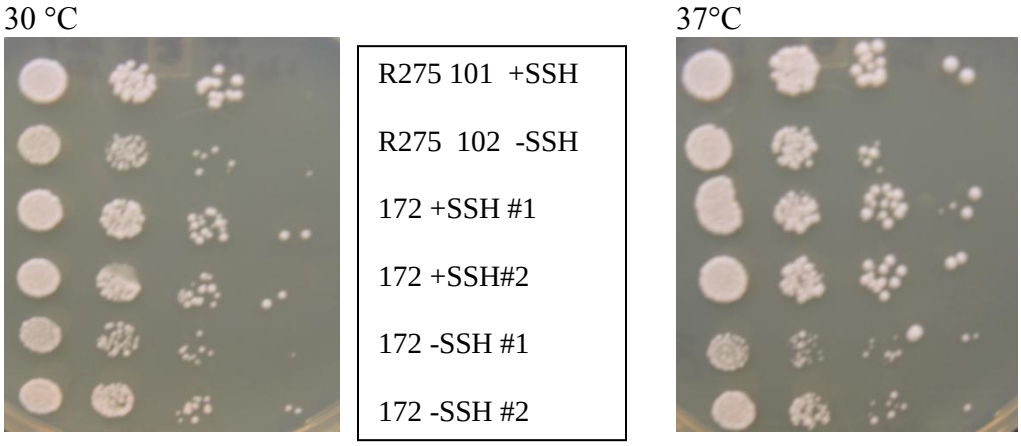

Figure-10: **Growth Analysis of Sec61 Mutant 172**. This mutant contains altered amino acids at positions C121A, C150A, I320C, C373A, and S72C. Left panel was incubated at 30°C and the right at 37°C for 2 days. Upper two rows shows WT strain R275 used as control.

Immunoblots to Verify Sec61 Expression

After confirming that some of the constructed mutants showed growth defects, Sec61 western blots were used to assay the cellular levels of the Sec61 protein. Whole cell lysates were prepared from the four mutant strains and the R275 positive control, loaded onto 10% SDS-PAGE gels, blotted to membrane, then analyzed with Sec61 and PGK antibodies. The upper band on each blot represents PGK, the middle band is full length V5 Sec61, and the lower band is mutant Sec61. The absence of V5 indicates whether WT Sec61 was successfully shuttled out of each mutant strain. PGK was used to detect an unrelated protein to determine whether non-target protein levels were affected by the Sec61 mutants. The immunoblots (**Figures 10, 11, and 12**) show that for WT strain R275, the absence of SSH lowered the level of Sec61 protein in one trial (Figure-

11) but increased it in a different trial (Figure-13). None of the mutant strains showed bands for V5, indicating that WT Sec61 (under the control of URA3) had been successfully shuttled out of those strains, and had been replaced with the mutant Sec61 (under the control of LEU2). Mutant 170 (Figure-11) showed a decrease in expression compared to WT containing Ssh in the same trial, but for this mutant with and without the presence of Ssh Sec61 was expressed at similar level. Comparing to WT without no Ssh this mutant had higher expression level. For mutant 172 (Figure-12), the expression level was higher in the presence of Ssh compared to without Ssh. This mutant closely resembles the WT expression in Figure 11. Mutants 138 and pEM 707 (Figure-13), both showed lower Sec61 levels with Ssh compared to without Ssh. Both of the mutants showed lower Sec61 level compared to WT containing Ssh. Without the presence of Ssh the Sec61 level was higher for 138 and the expression for pEM707 lower compared to WT. The purpose of these immunoblot was to show whether these mutant Sec61 are generally expressed in the yeast cells.

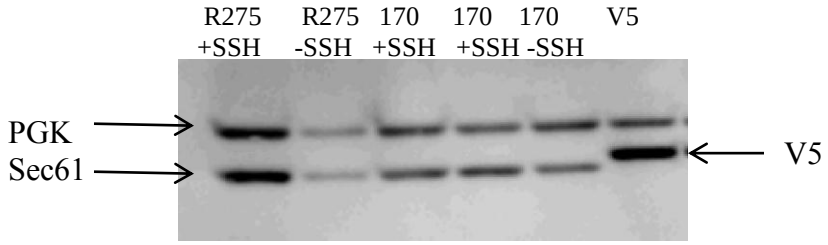

Figure-11: Western Blot Analysis of Mutant 170.

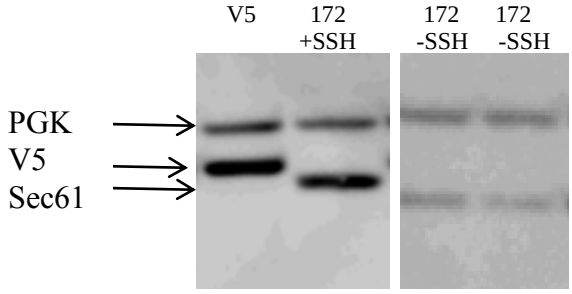

Figure-12: Western Blot Analysis of Mutant 172.

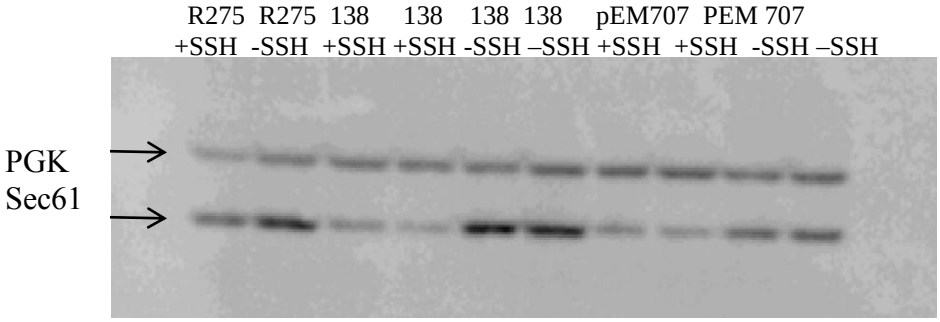

Figure-13: Western Blot Analysis of Mutants 138 and pEM707.

Due to time constraints, the accessibility of various substrates to the plug region of each Sec61 mutant was not determined. However, the overall results show that each mutant was successfully constructed and produced Sec61 protein which can be analyzed in the future.

DISCUSSION

In order to increase our understanding of the molecular mechanisms of Sec61 ER translocation channel opening, various Sec61 mutants were constructed. Successful mutagenesis was verified by sequence analysis, and Western blots demonstrated that each mutant expressed Sec61 protein. The mutants will be used in the future to determine the accessibility various substrates to the plug region.

The sites chosen for mutagenesis included cysteine residues at C121, C150, and C373, because these may be key residues important for accessibility in the plug region. Residue I320 was mutated because it is expected to allow subsequent labeling with maleimide with or without ribosome for future experiments. Sites M69 and S72 are at the pore region of the complex. The dilution assays of the four mutants showed growth defects, so these mutants could alter protein translocation. Comparing the mutants, 170 and 172 showed more effects on yeast growth, so the mutated cysteine residues, I320, and the residues in the pore region together could alter protein translocation.

The immunoblots confirmed the expression of Sec61 protein in each strain, and also showed that none of the mutant strains produced Sec61 V5 indicating the URA3-marked Sec61 plasmid was successfully shuffled out and was only expressing the LEU2-marked mutant Sec61.

Many problems were encountered in this project. A major problem occurring many times was a lack of any positive clones following transformation of ligated plasmid. Since most screened plasmids were negatives, the calf intestinal phosphatase procedure used to dephosphorylate the 5’ ends of the vector may have been inefficient,

allowing self-ligation without insert. In addition, one positive clone was lost after confirming the presence of insert with HindIII digestion, perhaps due to poor mini-preps and contamination. For the yeast transformations, on several occasions no colonies grew, possibly due to the presence of WT plasmid which does not allow growth on the selection plates.

Overall, the mutant strains created in this project will allow follow up assays to be performed to determine whether the mutants affect protein translocation, to determine whether Sec61 is in an open state conformation, and to determine the accessibility of substrates to the plug region. This represents the first step to our lab's long term goal of understanding the molecular mechanisms of translocation channel opening.

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